“Isolation and Growth Study of Coliform Isolated from Prayagraj in Response to Antimicrobial Peptide”

Project Report Submitted to Sister Nivedita University for Partial Fulfilment of The Requirement for The Degree of Master of Science in Microbiology

Submitted By

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This is to certify that

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has completed the project entitled **"Isolation and Growth Study of Coliform Isolated from Prayagraj in Response to Antimicrobial Peptide"** from January 2025 to May 2025, under the supervision of Dr. Kirat Kumar Ganguly, Assistant Professor, HOD Microbiology of this college, as part of her curriculum-based dissertation for the partial fulfilment of M.Sc. Degree in Microbiology from Sister Nivedita University.

I wish her career success.

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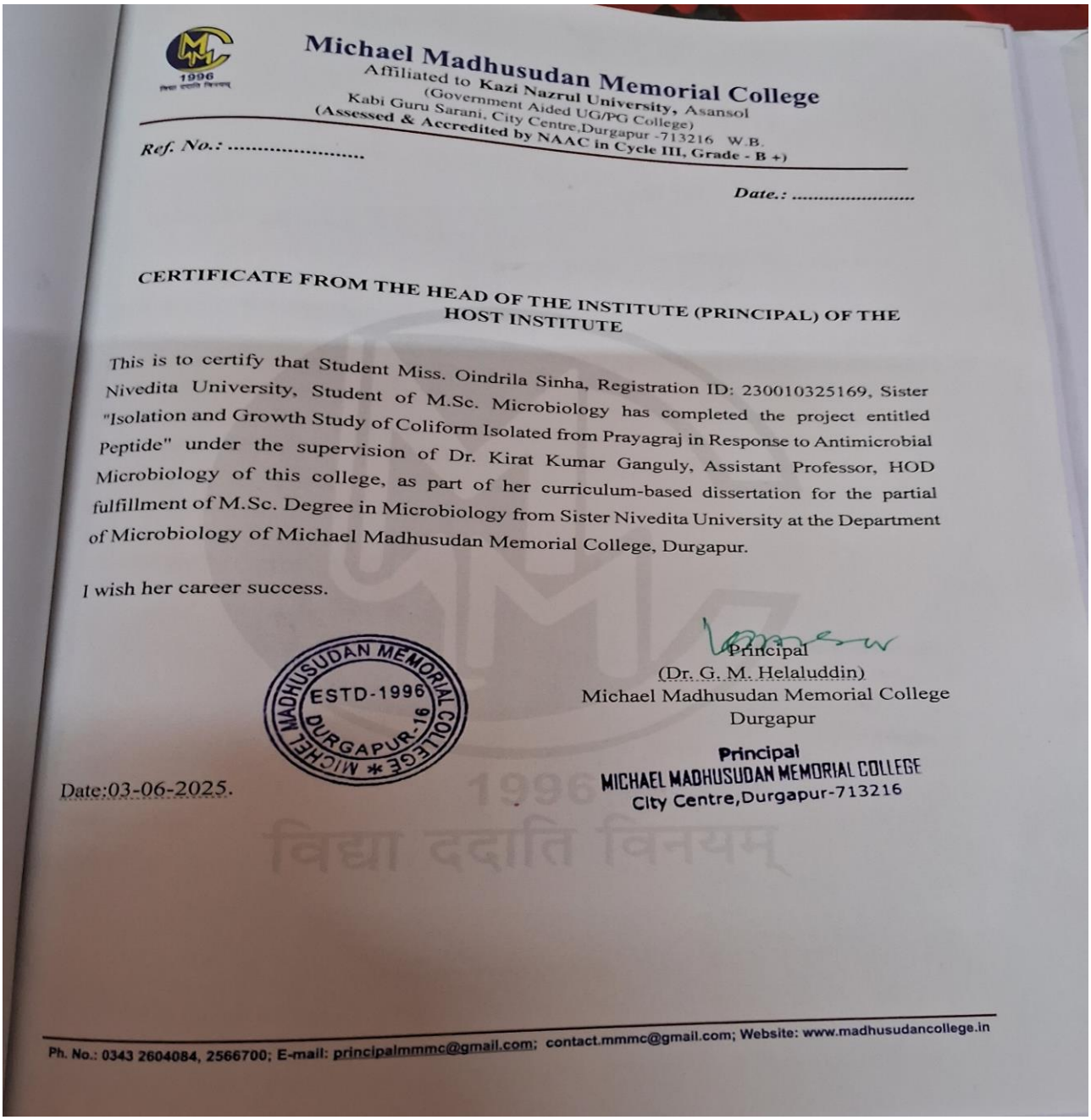
2nd June, 2025



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I wish her career success.

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CERTIFICATE FROM SUPERVISOR

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I wish her career success.

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Declaration By The Student

I hereby declare that the work which is being presented in this dissertation entitled "Isolation and Growth Study of Coliform Isolated from Prayagraj in Response to Antimicrobial Peptide" in partial fulfilment of the requirements for the award of the degree of Bachelors/Masters/Ph.D of Masters in Microbiology is an authentic record of my own work carried out during the period from January to May under the supervision of Dr. Kirat Kumar Ganguly, HOD and Assistant Professor of Department of Microbiology.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

I confirm that this dissertation has been checked for plagiarism using the plagiarism checking software Small SEO Tool. At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed. I herewith confirm that the dissertation has been less than 10% similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University.

I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations.

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To Whom It May Concern

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This is to certify that the project report titled "… Isolation and Growth Study of Coliform Isolated from Prayagraj in Response to Antimicrobial Peptide …", submitted by Mr./Ms. Oindrila Sinha….. (Registration No. 230010325169……. Enrolment No…2322211001032…….) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him/her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 6/6/25

Place: Newtown, Kolkata.

Signature of Head of the Department

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Abbreviations:-

NA:- Nutrient Agar Media.

NB:- Nutrient Broth Media.

EMB Agar:- Eosin Methylene Blue Agar Media.

Full Form of IMViC Test:-

I:- Indole Test.

M:- Methyl Red Test.

V:- Voges Proskauer Test.

C:- Citrate Utilization Test.

PBS:- Phosphate Buffered Saline.

PRYJ:- Prayagraj.

EDTA:- **Ethylenediaminetetraacetic acid.**

Abstract

The escalating threat of antibiotic resistance necessitates the exploration of alternative antimicrobial agents. This study focused on isolating and characterizing coliform bacteria from water samples collected in Prayagraj, India, and investigating the effect of an antimicrobial peptide (AMP) on their growth. For Example- Escherichia coli, a prevalent indicator of fecal contamination, was successfully isolated and identified using standard microbiological techniques and biochemical characterization. Growth kinetics of the isolated E. coli strain were established in vitro. Subsequently, the antimicrobial activity of a selected AMP was evaluated against the isolated E. coli using growth inhibition assays. The results demonstrated that the AMP exhibited significant inhibitory effects on the growth of the E. coli isolate in a dose-dependent manner. Further analysis, such as minimum inhibitory concentration (MIC) determination and time-kill assays, provided insights into the potency and mechanism of action of the AMP. This study highlights the potential of AMPs as promising alternatives to conventional antibiotics in combating coliform bacteria and underscores the need for further research into their application in water treatment and infection control.

Keywords:-

Pure Culture Isolation, EMB Agar Plating, Bacterial Inhibitory Zone, Biofilm Estimation. Antimicrobial Peptides.

Contents:

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Introduction:-

The presence of coliform bacteria in water sources poses a significant public health concern globally, particularly in regions with inadequate sanitation infrastructure. These microorganisms, often originating from fecal contamination, serve as indicators of potential pathogenic contamination and can lead to various waterborne diseases (WHO, 2017). Among the coliform group, Escherichia coli (E. coli) is a widely studied species due to its abundance and its association with both commensal and pathogenic strains capable of causing gastrointestinal and extraintestinal infections (Kaper et al., 2004). Prayagraj, a city in Uttar Pradesh, India, with its dense population and diverse water usage patterns, is susceptible to microbial contamination of its water resources. Understanding the prevalent coliform species in this region and their growth characteristics is crucial for assessing water quality and implementing effective control measures.

Antimicrobial peptides (AMPs) are a diverse class of naturally occurring molecules that exhibit broad-spectrum antimicrobial activity against bacteria, viruses, fungi, and even some parasites (Brogden, 2005). These peptides, typically short sequences of amino acids, often possess unique mechanisms of action, such as disrupting microbial cell membranes, which can make it more difficult for bacteria to develop resistance compared to conventional antibiotics that often target specific intracellular pathways (Hancock & Sahl, 2006). The potential of AMPs as alternative therapeutic agents and their possible applications in water disinfection have garnered significant attention in recent years.

Given the public health significance of coliform contamination in water sources like those in Prayagraj and the pressing need for novel antimicrobial strategies, this study aims to: (i) isolate and identify prevalent coliform bacteria from water samples collected in Prayagraj; (ii) characterize the in vitro growth kinetics of a selected isolate, and (iii) investigate the antimicrobial activity of a chosen antimicrobial peptide against the isolated bacterial strain. The findings of this research could contribute valuable insights into the microbial quality of water in the Prayagraj region and evaluate the potential of AMPs as a future strategy for controlling coliform contamination. The IMViC tests are a series of four biochemical tests used to identify and differentiate members of the Enterobacteriaceae family, especially Escherichia coli, Enterobacter, Klebsiella, etc.

Background:

The presence of coliform bacteria in water is a well-established indicator of fecal contamination and a potential risk to public health. Coliforms are a group of Gram-negative, rod-shaped bacteria that ferment lactose with the production of acid and gas when incubated at 35-37°C. While some coliforms are harmless inhabitants of the soil and vegetation, their presence in water typically suggests contamination with the feces of warm-blooded animals, including humans (APHA, 2017). Escherichia coli is the most common fecal coliform and its detection in water is a strong indicator of recent fecal contamination, signifying a higher risk of the presence of enteric pathogens such as Salmonella, Shigella, and various viruses and protozoa (Geldreich & Rice, 1987).

Prayagraj, situated at the confluence of the Ganges, Yamuna, and Saraswati rivers in Uttar Pradesh, India, is a densely populated urban center with significant religious and cultural importance. The city's water sources, including these rivers and groundwater, are subjected to various anthropogenic pressures, including discharge of untreated or partially treated sewage, agricultural runoff, and industrial effluents.

Antimicrobial peptides (AMPs) represent a promising class of molecules with the potential to overcome some of the limitations associated with conventional antibiotics. These naturally occurring peptides are part of the innate immune system of various organisms, exhibiting broad-spectrum antimicrobial activity through diverse mechanisms, often involving disruption of microbial cell membranes (Hancock & Chapple, 1999).

Research on AMPs has gained momentum in recent decades, with efforts focused on identifying novel peptides from natural sources, designing synthetic peptides with enhanced antimicrobial activity and stability, and exploring their potential applications in various fields, including medicine, agriculture, and environmental sanitation. Investigating the efficacy of AMPs against coliform bacteria isolated from specific geographical locations like Prayagraj is crucial for understanding their potential in addressing local water contamination issues and combating antibiotic resistance in this context.

Review of Literature:-

The study of coliform bacteria, particularly Escherichia coli, in water sources has been a significant area of research due to their role as indicators of fecal contamination and potential health risks. Numerous studies have documented the prevalence of coliforms in various water bodies across India, including the Ganges River, which flows through Prayagraj. For instance, a study by Singh et al. (2014) investigated the microbial quality of the Ganges River in the Allahabad region (now Prayagraj) and reported high levels of fecal coliforms, indicating significant pollution from domestic sewage and other anthropogenic activities.

Research has also focused on characterizing the antibiotic resistance profiles of coliform bacteria isolated from aquatic environments in India. A study by Gautam et al. (2016) examined the prevalence of antibiotic-resistant E. coli in the Yamuna River and found a high percentage of isolates resistant to multiple commonly used antibiotics. The presence of antibiotic-resistant coliforms in water sources poses a direct threat to public health as these bacteria can potentially transfer resistance genes to other pathogenic bacteria, complicating the treatment of infections.

While there is a considerable body of literature on coliform contamination in Indian water bodies and the general antimicrobial activity of various peptides, there is a relative scarcity of studies specifically focusing on coliform bacteria isolated from the Prayagraj region and their susceptibility to specific antimicrobial peptides. This study aims to bridge this gap by isolating and characterizing coliforms from Prayagraj water sources and evaluating the efficacy of a selected AMP against a representative isolate, E. coli. This research will contribute to a more localized understanding of water quality issues in Prayagraj and the potential of AMPs as a targeted antimicrobial strategy in this context.

Function and Mechanism:

The study encompasses two primary functional aspects: first, the isolation and characterization of coliform bacteria from water sources in Prayagraj, and second, the evaluation of the antimicrobial activity and potential mechanism of action of a selected antimicrobial peptide (AMP) against a representative isolate.

1. Function: Isolation and Growth Study of Coliform Bacteria

The primary function of this part of the study is to identify and cultivate coliform bacteria present in the water samples collected from Prayagraj. This involves:

Isolation: Employing selective and differential culture media to isolate coliform bacteria from the diverse microbial community present in the water samples. This typically involves media like MacConkey agar, which selects for Gram-negative bacteria and differentiates lactose fermenters (coliforms) from non-fermenters based on colony color.

Identification: Utilizing standard microbiological techniques and biochemical tests to identify the isolated coliform bacteria at least to the genus level, and ideally to the species level (e.g., Escherichia coli). This may involve Gram staining, motility tests, and a battery of biochemical assays such as the IMViC tests (Indole, Methyl Red, Voges-Proskauer, Citrate utilization) to differentiate between different coliform genera and species. Molecular methods, such as PCR amplification of specific genes, could provide more definitive identification.

Growth Study: Once a representative coliform isolate (e.g., E. coli) is obtained, its growth characteristics under standard laboratory conditions are investigated. This typically involves:

Growth Curve Determination: Culturing the isolate in a suitable liquid medium (e.g., nutrient broth) and measuring the bacterial population density (e.g., by spectrophotometry at regular intervals) over time. This allows for the determination of key growth parameters such as lag phase, exponential growth rate, generation time, stationary phase, and carrying capacity.

Optimization of Growth Conditions (Optional): Investigating the effect of various environmental factors (e.g., temperature, pH, nutrient concentration) on the growth rate and final yield of the isolate.

The information obtained from this part of the study establishes a baseline understanding of the prevalent coliform bacteria in Prayagraj water and the growth characteristics of a selected representative isolate, which is essential for evaluating the impact of the antimicrobial peptide.

2. Function: Antimicrobial Activity and Mechanism of Antimicrobial Peptide

The second primary function of the study is to assess the effectiveness of a chosen antimicrobial peptide against the isolated E. coli strain and to gain preliminary insights into its mechanism of action. This involves:

Antimicrobial Activity Evaluation: Determining the ability of the AMP to inhibit the growth of the E. coli isolate. This typically involves:

Minimum Inhibitory Concentration (MIC) Assay: Determining the lowest concentration of the AMP that visibly inhibits the growth of the E. coli culture after a specific incubation period. This is a quantitative measure of the peptide's potency.

Growth Inhibition Assays (e.g., Colony Forming Unit (CFU) assays): Quantifying the reduction in viable bacterial cells upon exposure to different concentrations of the AMP over time.

Time-Kill Kinetics: Monitoring the change in viable bacterial counts over time at a specific concentration(s) of the AMP to understand the rate and extent of killing.

By elucidating the function and mechanism of the chosen AMP against the locally isolated E. coli, this part of the study contributes to the understanding of its potential as a novel antimicrobial agent to combat coliform contamination and antibiotic resistance in the Prayagraj context.

Occurrence and Diversity:-

Coliform bacteria are ubiquitous in the environment, particularly in the gastrointestinal tracts of warm-blooded animals, including humans. Their presence in water sources is a well-established indicator of fecal contamination. The occurrence and diversity of coliforms in aquatic environments are influenced by various factors, including:

Source of Contamination: The primary source of coliforms in water is fecal matter from human and animal waste. This can enter water bodies through untreated or inadequately treated sewage discharge, agricultural runoff containing animal manure, and direct deposition from wildlife. In densely populated areas like Prayagraj, the discharge of domestic sewage into rivers like the Ganges and Yamuna is a significant contributor to coliform contamination (Singh et al., 2014; Current Affairs, 2025).

Environmental Factors: Factors such as water temperature, pH, nutrient availability, and sunlight exposure can influence the survival and persistence of coliform bacteria in water. Studies have shown higher occurrences of coliforms at warmer temperatures (R Discovery).

Seasonal variations in rainfall and water flow can also affect the concentration and distribution of these bacteria (Discovery Researcher Life).

Water Source Type: The occurrence and diversity of coliforms can vary between different types of water sources, such as rivers, groundwater, and treated drinking water. Surface waters are generally more susceptible to fecal contamination compared to properly treated groundwater sources. However, breaches in treatment or distribution systems can lead to coliform contamination even in drinking water (ResearchGate).

Studies focusing on the water quality of the Ganges and Yamuna rivers in the Prayagraj region have consistently reported high levels of total and fecal coliforms, often exceeding the permissible limits for bathing and drinking water (Current Affairs, 2025; India Today, 2025; Deccan Herald, 2025). These findings highlight the significant fecal pollution in these religiously important rivers, posing a substantial public health risk to the millions of people who use these waters for various purposes, including bathing during the annual Magh Mela and the Maha Kumbh Mela (Times of India, 2025).

Therefore, understanding the specific occurrence and diversity of coliform bacteria in Prayagraj water sources, including their antibiotic resistance profiles, is crucial for assessing the local water quality and developing effective strategies to mitigate contamination and protect public health. This study, by isolating and characterizing coliforms from Prayagraj and investigating the potential of antimicrobial peptides, aims to contribute to this localized understanding.

Social Applications:

The research topic "Isolation and growth study of any coliform bacteria isolated from Prayagraj with antimicrobial peptide" holds significant social applications, particularly in the context of public health, environmental management, and the fight against antibiotic resistance:

Improved Water Quality Monitoring and Management in Prayagraj: Identifying the specific types and levels of coliform bacteria prevalent in Prayagraj's water sources (like the Ganges and Yamuna rivers) provides crucial data for local authorities and environmental agencies. This information can be used to:

Assess the extent of fecal contamination: Pinpointing areas with high coliform counts can help identify pollution sources (e.g., untreated sewage discharge, agricultural runoff).

Inform public health advisories: High coliform counts can trigger warnings to the public about potential health risks associated with using the water for drinking, bathing, or other purposes, especially during large gatherings like the Magh Mela and Kumbh Mela.

Combating Antibiotic Resistance: Studying the susceptibility of locally isolated coliform bacteria to antimicrobial peptides (AMPs) can contribute to the global effort against antibiotic resistance. This research can be:-

Public Health Protection and Disease Prevention: By understanding the microbial quality of water and exploring new ways to combat bacterial contamination, this research directly contributes to protecting public health. This can lead to:

Reduced incidence of waterborne diseases: Effective monitoring and treatment of coliform contamination can lower the risk of diarrheal diseases, cholera, typhoid fever, and other waterborne illnesses, particularly among vulnerable populations.

Improved sanitation practices: The findings can underscore the importance of proper sanitation and wastewater management in preventing fecal contamination of water sources.

Increased productivity: A healthier population experiences less morbidity, leading to increased productivity and economic growth.

In conclusion, the proposed research has significant social applications by providing crucial insights into the microbial quality of water in a highly populated and religiously significant region, contributing to the fight against antibiotic resistance, and ultimately aiming to protect public health and improve the overall well-being of the community in Prayagraj.

Hypothesis:-

Coliform bacteria isolated from environmental samples in Prayagraj will show differential susceptibility to selected antimicrobial peptides (AMPs), with some strains exhibiting significant growth inhibition and others showing resistance, indicating variation in AMP effectiveness against local coliform populations.

Objectives

- To isolate and identify coliform bacteria from water samples collected in Prayagraj.
- To perform morphological, biochemical, and molecular characterization of the isolated coliform strains.

- To assess the baseline growth patterns of the isolated coliform strains under standard laboratory conditions.
- To expose the coliform isolates to selected antimicrobial peptides and evaluate their growth response (e.g., inhibition zones, Biofilm estimation).
- To compare the susceptibility of different coliform strains to the antimicrobial peptides tested.

Bacterial Pure Culture Isolation:-

Materials Required:-

1) Two Petridishes.
2) One Conical Flask.
3) Inoculation Loop.
4) Autoclave.
5) Laminar Airflow.
6) Incubator.

Performing Bacterial Gram Staining:-

Materials Required:-

1) A clear and clean slide.
2) A Micropipette.
3) A Light Microscope.
4) An Inoculation Loop.
5) A spirit Lamp.
6) Alcohol.
7) Distilled Water.
8) Tissue Paper.
9) Crystal Violet.
10) Gram's Iodine.
11) Decolorizer.
12) Safranin.

EMB Agar Plating:-

Materials Required:-

1) Petridishes.
2) Conical Flasks.
3) Glass Spreader.
4) Inoculation Loop.

Composition of EMB Agar:-

1) Distilled Water:- 50ml.
2) EMB Agar:-1.798gm.

Nutrient Agar Medium Plating:-

Materials Required:-

1) One Conical Flask.
2) Two Petridishes.
3) Autoclave.
4) Laminar Airflow.
5) Fridge.
6) Parafilm.
7) Incubator.

Composition of Nutrient Agar:-

1) Distilled Water:- 50ml.
2) Nutrient Agar:- 1.4gm.

Experiment of Bacterial Inhibitory Zone:-

Materials Required:-

1) Petridishes.
2) Steriled PBS.
3) Microcentrifuge tubes.
4) Autoclave.
5) Incubator.
6) Laminar Airflow.
7) Vortex.
8) Borer.

9) Glass Spreader.
10) Micropipette.
11) Alcohol.

IMViC Test:-

Materials Required:-

1) Test Tubes.
2) Indicators and Reagents.
3) Inoculation Loop.
4) Laminar Airflow.
5) Autoclave.
6) Incubator.
7) Big Beaker.

Composition of Media:-

1) Indole Test (IT):- a) Tryptone= 0.2gm
2) Methyl Red Voges Proskauer Test (MR-VP):-
   A) MR-VP Broth:-
      i) Peptone= 0.14gm
      ii) Dextrose= 0.1gm
      iii) Dipotassium Phosphate= 0.1gm.
3) Voges Proskauer Test (VP):-
   A) MR-VP Broth:-
      i) Peptone= 0.14gm
      ii) Dextrose= 0.1gm
      iii) Dipotassium Phosphate= 0.1gm.
4) Citrate Utilization Test (CU):-
   A) Simmons' Citrate Agar:-
      i) Sodium Citrate= 0.04gm
      ii) Ammon0.04Dihydrogen Phosphate= 0.02gm
      iii) Dipotassium Phosphate= 0.02gm
      iv) Magnesium Sulfate= 0.004gm
      v) Sodium Chloride= 0.1gm
      vi) Bromothymol Blue= 0.016gm

vii) Agar Powder= 0.3gm.

Indicators and Reagents:-

1) Methyl Red Voges Proskauer Test (MRVP):- Methyl Red Indicator= 0.01gm Methyl Red + 10ml Ethanol + 90ml Distilled Water.
2) Voges Proskauer Test (VP):-

Barritt's A Reagent= 5% alpha- naphthol dissolved in absolute alcohol. (0.5gm alpha-naphthol + 10ml Alcohol).

3) Barritt's B Reagent= 40% KOH Solution. (4gm KOH + 10ml Distilled Water).

Bacterial Inoculation in Nutrient Broth Test Tubes:-

Materials Required:-

1) 5 Test tubes.
2) Inoculation Loop.
3) Spirit Lamp.
4) Autoclave.
5) Incubator.
6) Laminar Airflow.

Composition of Nutrient Broth:-

a) Nutrient Broth= 0.65gms.
b) Distilled Water= 50ml.

Biofilm Estimation:

Materials Required:

1) Crystal Violet.
2) Empty Conical Flask.
3) Autoclave.
4) Distilled Water.

Bacterial Plasmid DNA Isolation:

Materials Required:-

1) Microcentrifuge.

2) Microcentrifuge Tubes.
3) Water Bath (37°C).
4) Automatic Micropipettes with tips.
5) 95-100% Isopropanol Ice.

Buffers and Solutions:-

a) Alkaline Lysis Solution I.
b) Alkaline Lysis Solution II.
c) Alkaline Lysis Solution III.
d) Antibiotic for plasmid selection.
e) Ethanol.
f) Phenol:- Chloroform.
g) STE.
h) TE containing 20ug/ml RNAse A.

Recipes for Buffers, Solutions and Media:-

1) Alkaline Lysis Solution I:-
    a) 0.9gm Glucose.
    b) 0.394gm Tris-Cl.
    c) 3.72gm EDTA.
2) Alkaline Lysis Solution II:-
    a) 4gm NaOH.
    b) 1% SDS.
3) Alkaline Lysis Solution III:-
    a) 0.04gm Potassium Acetate.
    b) 100ml Distilled Water.
    c) Glacial Acetic Acid. (11.5ml.)
    d) 28.5ml H2O.
    e) EDTA.
    f) Glycerol.
    g) LB Media.
    h) NaCl.
    i) NaOH.
    j) Potassium Acetate.

k) SDS.

l) STE.

m) TE.

n) Tris- Cl.

Methods/ Procedure of Experiment- Bacterial Pure Culture Isolation:-

Step1:- Prepare 50ml Nutrient Agar Media in a 250ml Conical Flask. (We would take 1gm of Nutrient Agar in 50ml Distilled Water.)

Step2:- Then cotton plug and wrap 250ml Conical Flask containing 50ml Nutrient Agar Media and also wrap 2 petridishes with big paper and autoclave these at 121°C at 15psi.

Step3:- After autoclave, take out these things and keep these inside Laminar Airflow.

Step4:- Pour Plate:- Then pour this Nutrient Agar Media in these 2 petridishes and put off the lid of Laminar Airflow and solidify this Media in these 2 petridishes for 15 minutes and discard this empty Conical Flask.

Step5:- Streak Plate:- After Nutrient Agar Media gets solidified, then burn spirit lamp and dip Inoculation Loop in alcohol and incinerate the loop and take Inoculum from this plate (PRYJ; 11/03) and do Streaking in Plate1. Again incinerate the loop and do streaking in Plate2 and then keep these 2 Petridishes inside Incubator at 30°C for 24 hours.

Experiment- Performing Bacterial Gram Staining:-

Procedure:-

Now, we will do Gram Staining by taking the pure culture or bacterial colony cells or bacterial inoculum from Streaked Plate1. Steps of Bacterial Gram Staining are:-

Step1:- Take a slide and wash it in tap water. Then take a clean tissue paper and dip in alcohol and rub on the slide and clean properly.

Step2:- Then take distilled water with the help of 200ul Micropipette and put 2 drops of distilled water on the middle of that clean slide.

Step3:- Burn on spirit lamp in Laminar Airflow and take an Inoculation Loop and incinerate the loop in spirit lamp until the loop becomes red hot and then dip in alcohol and repeat this totally 3 times.

Step4:- Then gently take less inoculum from that Streaked Plate1 and do smear on the slide by spreading the Bacterial inoculum with distilled water and also separating those Bacterial cells with the help of Inoculation Loop.

Step5:- Then incinerate that loop and dip in alcohol and then heat fix that slide on the spirit lamp for 5 minutes.

Step6:- After that, do air dry that slide until the smeared inoculum gets totally dry.

Step7:- After completing air dry, we will give Crystal Violet on the inoculum of slide and remain for 1 minute. Then we will wash out Crystal Violet gently by putting few drops of tap water on the slide.

Step8:- Then we will give Gram's Iodine on the slide and wait for 50 seconds. Then we will wash out Gram's Iodine gently by putting Decolorizer on that slide.

Step9:- At last, we will give Safranin on that inoculum of slide and wait for 1 minute.

Step10:- After 1 minute, we will wash out Safranin gently from that slide with the help of tap water and then we will air dry that slide until the slide gets dry.

Step11:- After drying of slide, we will keep that slide under Light Microscope.

Experiment- EMB Agar Plating:-

Procedure:-

Step1:- We will prepare 50ml Nutrient Broth in 100ml Conical Flask. Then we will also prepare 50ml EMB Agar in 100ml Conical Flask.

Step2:- Then we will cotton plug the Conical Flask having EMB Agar and wrap it with paper. Then we will wash properly 5 test tubes with tap water and in each 5 test tubes we will give 10ml Nutrient Broth with the help of measuring glass cylinder from that 100ml Conical Flask. Then, we would mark 5 Test tubes as:- NB1 (Inoculated), NB2 (Inoculated), NB3 (Inoculated), NB4 (Inoculated), and NB5 (Blank) Test Tubes.

Step3:- Then we will cotton plug those 5 test tubes and wrap those with paper and also wrap PBS, Glass Spreader and 2 petridishes with paper and put these all things inside Autoclave at 121°C for 15psi.

Step4:- After Autoclave, take out all those things and keep in Laminar Airflow.

Step5:- Then pour EMB Agar in 2 plates and get them solidified. After EMB Agar gets solidified, then take inoculum from Streak Plate1 (PRYJ; 11/03) and Streak the two EMB Agar plates and label the two petridishes as EMB Plate1 and EMB Plate2 and then keep those 2 EMB Agar plates in Incubator and wrap those 5 test tubes having Nutrient Broth with paper and keep those in Fridge. Then also wrap Streak Plate1 with Parafilm and keep it in Fridge.

Experiment- Nutrient Agar Medium Plating:-

Procedure:-

Step1:- Prepare 50ml Nutrient Agar and autoclave a 250ml Conical flask containing this 50ml Nutrient Agar, also autoclave two petridishes at 121°C at 15 psi.

Step2:- After autoclave, we will pour Nutrient Agar Medium in these 2 petridishes and get them solidified.

Step3:- After solidifying Nutrient Agar Medium, we will label those 2 plates as- Nutrient Agar Plate1, Nutrient Agar Plate2 and keep those 2 plates inside Incubator at 30°C.

Experiment- Bacterial Inhibitory Zone:-

Procedure:-

Step1:- After few days, we would take out those 2 Nutrient Agar Plates, Steriled PBS and Previous Nutrient Agar Streaked Plate (PRYJ, 11/03) and keep those in Laminar Airflow.

Step2:- Then, take a sterilized microcentrifuge tube and put 900ul sterilized PBS inside that tube with the help of Micropipette.

Step3:- Then incinerate Inoculation Loop and take inoculum from that Nutrient Agar Streaked Plate (PRYJ, 11/03) with the help of loop and inoculate it inside that tube and Vortex that tube, so that PBS mixes properly with that inoculum.

Step4:- Then take that mixed inoculum and put in the two Nutrient Agar Plates with the help of 100ul Micropipette and dip glass Spreader in Alcohol and incinerate Spreader and then gently spread the mixed inoculum on the surface of Nutrient Agar Medium in both 2 Nutrient Agar Plates.

Step5:- Then dip borer in alcohol and incinerate properly and then bore (means make 4 holes in both of the two Nutrient Agar Plates.)

Step6:- We made the precipitate by taking bacterial sample from Bakreshwar. Then take 50ul Micropipette and put the precipitate in 1 hole of Nutrient Agar Plate1 and put precipitate in 1 hole of Nutrient Agar Plate2.

Step7:- Then put alcohol in 1 hole of Nutrient Agar Plate1 and Nutrient Agar Plate2. Then, gently keep those two Nutrient Agar bored Plates inside Incubator at 30°C for 24 hours. Then wrap PBS and that Streaked Nutrient Agar Plate (PRYJ, 11/03) with Parafilm and keep those inside Fridge at 4°C.

Experiment- IMViC Test:-

The IMViC tests are a series of four biochemical tests used to identify and differentiate members of the Enterobacteriaceae family, especially Escherichia coli, Enterobacter, Klebsiella, etc.

Procedure:-

Step1:- As we saw Green Metallic Shean of Bacterial Colonies (Coliform Bacteria) in both of the two EMB Agar Plates previously, so we would perform IMViC Test in Lab. We took 20ml Distilled Water in 4 Conical Flasks and measured all different medias and mixed with distilled water in each 4 Conical Flasks.

Step2:- Then we took 8 Test tubes and at first put 10ml of different media in each test tubes with the help of small 10ml Measuring Glass Cylinder.

Step3:- Then, cotton plug all Test tubes and put those in a big beaker and wrap it with a big paper and autoclave it at 121°C for 15 psi.

Step4:- After autoclave, keep it in Laminar Airflow and inoculate Test tubes labelled as:- IT(Inoculated), MRVPT(Inoculated), VPT(Inoculated), CUT(Inoculated) with the help of Inoculation Loop by taking Green Metallic Shean of many Bacterial colonies in EMB Agar Streaked Plate1. We know that, we have two Test tubes like one Test tube is having Inoculation and another Test tube is having no inoculation for each 4 tests such as:- Indole Test (IT), MRVP Test, VP Test and Citrate Utilization Test (CU), etc.

Step5:- At last case, that is in case of Citrate Utilization Test (CU), we would slant both of the two Test tubes for 30 minutes. Then we will take culture from that EMB Agar Plate1 and would gently Streak inside the slant of only one Test tube labelled or marked as CUT (Inoculated) with the help of Inoculation Loop and then we will keep all those test tubes inside Incubator at 37°C for 48 hours.

Step6:- On next day, we would take out those test tubes and keep in Laminar Airflow and prepare some Indicators and Reagents like- Methyl Red Indicator for Methyl Red Test, and Barritt's A Reagent, Barritt's B Reagent for Voges Proskauer Test (VP Test) in Lab.

Experiment- Bacterial Inoculation in Nutrient Broth Test Tubes:-

Procedure:-

Step1:- Previously we prepared Nutrient Broth and distributed 10ml of Nutrient Broth in each 5 test tubes and autoclaved it. Then, we would inoculate those 4 Nutrient Broth Test Tubes by taking inoculum from EMB Streaked Plate1 (in which we saw Metallic Green Shean of Coliform Bacterial Colonies) with the help of Inoculation Loop.

Step2:- We would keep one Test tube as Blank and wouldnot inoculate in that Blank Nutrient Broth Test Tube.

Step3:- Then we would keep those 5 Nutrient Broth Test Tubes inside Incubator at 37°C for 48 hours.

Experiment- Biofilm Estimation:

Biofilm estimation refers to the process of quantifying the presence and amount of biofilm formed by microbial cells on a surface. It is a crucial step in microbiology research, clinical diagnostics, and industrial settings to evaluate microbial adherence, biofilm-forming ability, and effects of antimicrobials.

Common Methods of Biofilm Estimation:

Crystal Violet Assay (CV Assay).

Principle: Crystal violet stains the biomass (cells and extracellular matrix) of the biofilm.

Procedure:-

Step1:- On the same day, we would again prepare 100ml Nutrient Broth in 250ml Conical Flask and will cotton plug it and wrap it with paper and will also cotton plug and wrap another empty 100ml Conical Flask with paper and autoclave these two Conical Flasks at 121°C for 15 psi.

Step2:- After Autoclave, we would keep those inside Laminar Airflow. Then we would perform Biofilm Estimation in Lab, means we would pour Nutrient Broth Media from all 4 Nutrient Broth Inoculated Test Tubes inside that empty 100ml Conical Flask, but some Bacterial cells remain inside those Test tubes.

Step3:- Then, we would take a 100ul tip Micropipette and put 4 drops of Crystal Violet in each 4 empty test tubes and then we would put 100ml distilled water in those 4 Test tubes marked as NB1(Inoculated), NB2(Inoculated), NB3(Inoculated), NB4(Inoculated) Test Tubes.

Step4:- Then we would observe that the Bacterial cells come in contact with Crystal Violet. We would keep these for 15 minutes.

Step5:- After 15 minutes we would throw that Crystal Violet having Bacterial cells in those 4 Test tubes to the basin and keep all 4 Test tubes on Test Tube Rack.

Experiment- Bacterial Plasmid DNA Isolation:-

Plasmid is a mostly double stranded, circular extra chromosomal DNA of bacterium. It is used in recombinant DNA experiments to clone genes from other organisms and make large quantities of their DNA.

Procedure:-

Step1:- Few days before we inoculated in one conical flask containing Nutrient Broth. After few days, we would take out the Conical Flask from Incubator. Now, we would work with this Inoculated Nutrient Broth Media having many Bacterial colonies.

Step2:- Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microfuge. Store the unused portion of the original culture at 4°C.

Step3:- Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

Step4:- Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.

Step5:- Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents well by inverting the tube . Do not vortex. Store the tube in ice.

Step6:- Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube in ice for 3-5 minutes.

Step7:- Centrifuge the bacterial lysate for 5 minutes at maximum speed at 4°C in a microfuge. Collect the supernatant to a fresh tube.

Step8:- (Optional) Add equal volume of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.

Step9:- Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.

Step10:- Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.

Step11:- Discard the supernatant by aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kim wipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.

Step12:- Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.

Step13:- Remove all of the supernatant by aspiration. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.

Step14:- Remove any beads of ethanol from the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes).

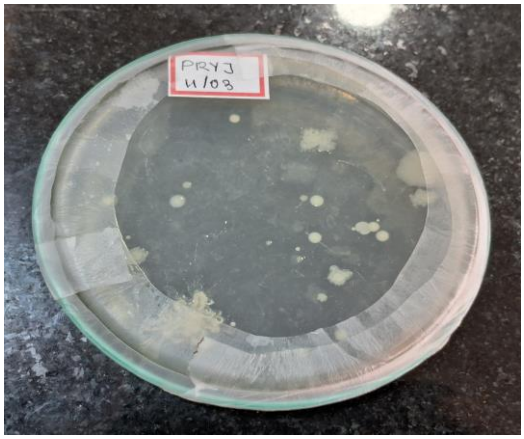
Step15:- Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds and store the DNA at -20°C.

Step16:- After that we would load the sample and then we would apply electricity for 1hour 30minutes to pass through the loaded samples. Then we would see or observe the result by putting the tray inside UV Transilluminator.

Result of Experiment- Bacterial Pure Culture Isolation:-

After 1 day, we will see that there is bacterial colony growth in Streaked Plate1 and in Streaked Plate2 there is fungal growth but no bacterial colony growth is there, so Streaked Plate2 is rejected and discarded as we will work with only bacterial colonies. So, we will work with only Streaked Plate1.

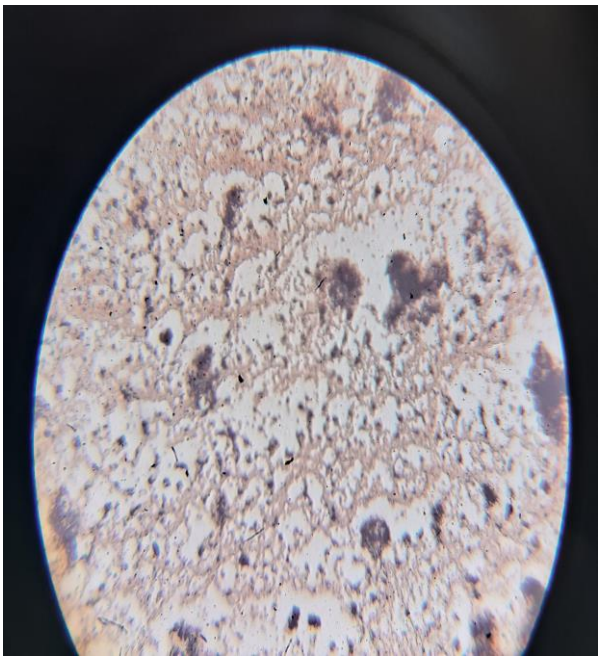
These are the Pictures of Petridishes having Prayagraj sample, Nutrient Agar Streaked Plate 1 and 2.



Result of Experiment- Performing Bacterial Gram Staining:-

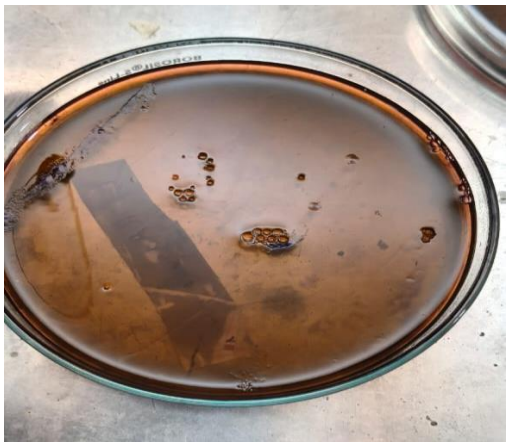
After performing Bacterial Gram Staining, we will keep the slide under Light Microscope at 40X and observe that there are proper and clear presence of Bacterial Colonies on that slide and those pictures are clearly given below. So we understand from here, that the real presence of Bacterial Colonies on that plate labelled as (PRYJ; 11/03) from which we have taken Bacterial Inoculum and Streaked on another plate (Streaked Plate1) and from this plate we have again taken Bacterial Inoculum and put on a slide, performed Gram Staining on it and saw the presence of Bacterial Colonies on that slide under Light Microscope. Then we will discard that slide and wrap that Streaked Plate1 with Parafilm and keep it inside Fridge at 4°C.

Picture of Bacterial Gram Staining seen under Microscope at 40X.



Result of Experiment- EMB Agar Plating:-

1) After few days, we will see that the growth of Bacterial Colonies has taken place in both the two EMB Streaked Plates. But in EMB Plate2, less Bacterial colony growth has taken place and in EMB Plate1, maximum Bacterial colony growth has taken place. We can see the Green Metallic Shean in both of the two EMB Streaked Plates which really proves us the presence of those bacterial colonies in 2 petridishes. So, we will wrap these 2 EMB Streaked Plates with Parafilm and keep these inside Fridge at 4°C. Pictures of EMB Streaked Plates 1 and 2 having Green Metallic Shean of Bacterial Colonies.



Result of Experiment- Nutrient Agar Medium Plating:-

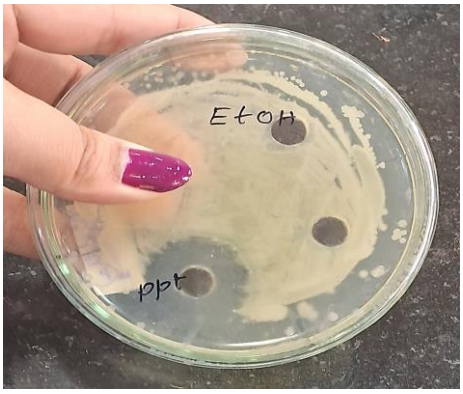
We can also see that there is no contamination in both the two Nutrient Agar Medium Plates. So, we will wrap these two Nutrient Agar Plates with Parafilm and keep these inside Fridge at 4°C.

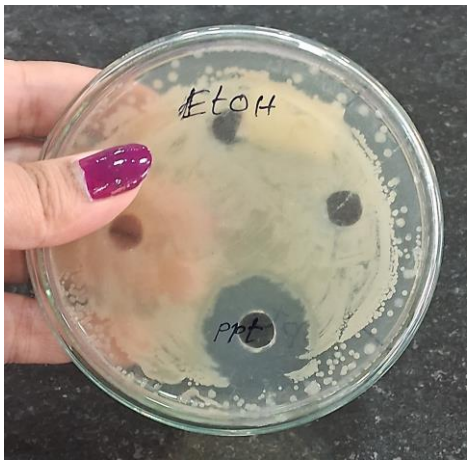
Result and Observation of Experiment of Bacterial Inhibitory Zone:-

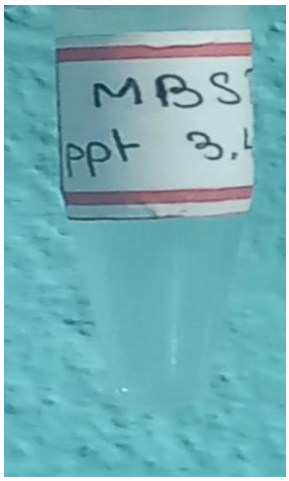
On next day, we would take out those 2 Nutrient Agar Bored Plates and see that there are clear and proper Inhibitory Zone around the circle of both Nutrient Agar Plate1 and Nutrient Agar Plate2 in which we gave Precipitate on the previous day. But amazingly no inhibitory zone was there around other circle in which we gave Alcohol in both Nutrient Agar Plate1 and Nutrient Agar Plate2. In other two holes of Nutrient Agar Plate1 and 2, we also saw no inhibitory zone in which we gave nothing. The presence of many Bacterial colonies were also seen in these both of the two Nutrient Agar Bored Plates. Then we would measure the radius of Inhibitory Zone around the circle of precipitate with the help of a plane or normal scale in both of the 2 Nutrient Agar bored plates and we would note the measurement (in cm) of radius of Inhibitory Zone of circle of precipitate in 2 of the Nutrient Agar Bored Plates and we would also calculate the average radius of Inhibitory Zone of circle of precipitate in both of the 2 Nutrient Agar bored plates. We can also understand that after giving inoculum and doing spread plate in both of the two Nutrient Agar plates, the bacterial colonies which are present in these 2 Nutrient Agar Plates having Inhibitory Zone, have less power than other bacteria which is taken from Bakreshwar and with this powerful bacteria we made the Precipitate and we gave this Precipitate in bored holes of both Nutrient Agar Plates. Then we got inhibitory zone in 1 hole of Nutrient Agar Plate 1 and 2, where we gave Precipitate. This

precipitate is formed as the more powerful bacteria taken from Bakreshwar eats or kills the less powerful bacteria present in the 2 Nutrient Agar Plates. We can see that when alcohol is given in 1 bored hole of 2 Nutrient Agar Plates, then no inhibitory zone forms, as alcohol has less power than the bacteria present in that 2 Nutrient Agar Plates. So, alcohol can't eat or kill that bacteria and we can understand that the bacteria of Bakreshwar is most powerful than other bacteria, and alcohol present in 2 Nutrient Agar Plates. Then we would wrap those 2 Nutrient Agar bored plates with parafilm properly and keep those 2 Nutrient Agar bored plates inside Fridge at 4°C. Here is the Measurement of Radius of Inhibitory Zone and Measurement of Average Radius of Inhibitory Zone of 2 Nutrient Agar Plates:-

Plate Type	**Radius1**	**Radius2**	**Radius3**	**Radius4**	**Average Radius of Bacterial Inhibitory Zone.**
Nutrient Agar Plate1	0.16cm	0.18cm	No	No	0.17cm
Nutrient Agar Plate2	1.2cm	0.16cm	0.16cm	0.17cm	0.4225cm





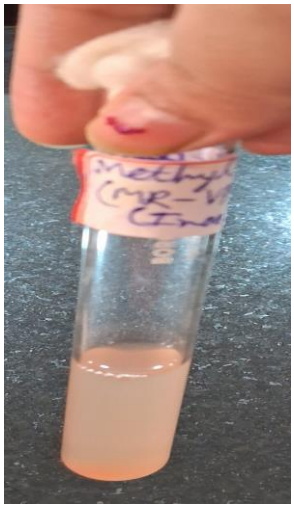


Pictures of Precipitate Sample and Bacterial Inhibitory Zone in Nutrient Agar Plate 1 and 2.

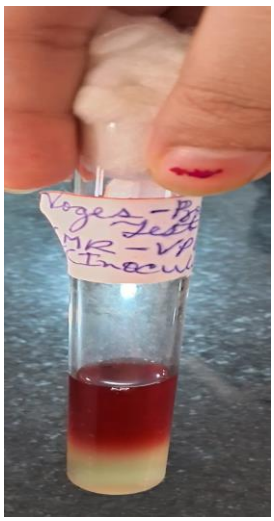
Result and Observation of Experiment- IMViC Test:-

1) We would give 5 times Kovac's Reagent in both IT(Inoculated and non-Inoculated test tubes). Then we would see same Deep Greenish Colour in both Test Tubes which denotes Indole Test as Negative Test. Pictures of Indole Test.

2) Then we would give 5 times of Methyl Red Indicator in both MRVP(Inoculated and non- Inoculated test tubes). Then we would see same yellow colour in both Test Tubes which shows MRVP Test as Negative Test.



3) Then we would give 10 drops of Barritt's A Reagent and 3 drops of Barritt's B Reagent in both VP(Inoculated and non- Inoculated test tubes). Then we would see a half layer of Deep Red Colour at upper part and yellow colour at lower part in VP Inoculated Test Tube, which shows that VP Test is a Positive Test. Whereas in VP Non- Inoculated Test Tube, we would see that the colour changes to Copper Red Colour.



4) Here, no Indicator or Reagent is required in both Citrate Utilization (Inoculated and Non- Inoculated Test tubes). On next day, that is on (29/4/25) we would take out both Citrate Utilization (Inoculated and Non- Inoculated Test tubes), we would see that no colour changes in Slant Citrate Utilization (Inoculated and Non-Inoculated Test Tubes). So, it denotes that Citrate Utilization Test is having Negative Test as both Citrate Utilization Inoculated and Non- Inoculated Test Tubes are having same Greenish Colour and this colour doesn't change after taking out these two Test tubes from Incubator.

Table of IMViC Test:-

Test	Positive Result	Negative Result
Indole(Inoculated)	No.	Green colour appears, so it is Negative Test.
Indole(Non- Inoculated)	No.	Green colour appears, so it is Negative Test.
Methyl Red(Inoculated)	No.	Yellow colour appears, so it is a Negative Test.
Methyl Red(Non-Inoculated)	No.	Yellow colour appears, so it is a Negative Test.
Voges Proskauer(Inoculated)	Red Colour Ring at upper layer. So, it is Positive.	No.
Voges Proskauer(Non-Inoculated)	No.	Copper colour appears, so it is Negative Test.
Citrate Utilization(Inoculated)	No.	No bacterial growth, the medium remains same Deep Green colour. So it is Negative Test.
Citrate Utilization(Non-Inoculated)	No.	No bacterial growth, the medium remains same Deep Green colour. So it is Negative Test.

Result and Observation of Experiment- Bacterial Inoculation in Nutrient Broth Test Tubes:-

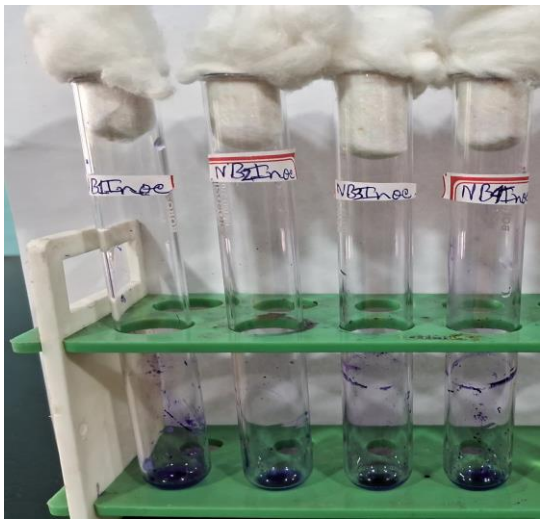
After few days, we would take out those 5 Nutrient Broth Test Tubes and see that perfect Bacterial colonies are present inside all 4 Nutrient Broth Test Tubes marked as- NB1(Inoculated), NB2(Inoculated), NB3(Inoculated), NB4(Inoculated) Test Tubes. But we can see clearly that contamination has taken place in NB5(Blank) Test Tube, so we would discard this NB5 (Blank) Test Tube.

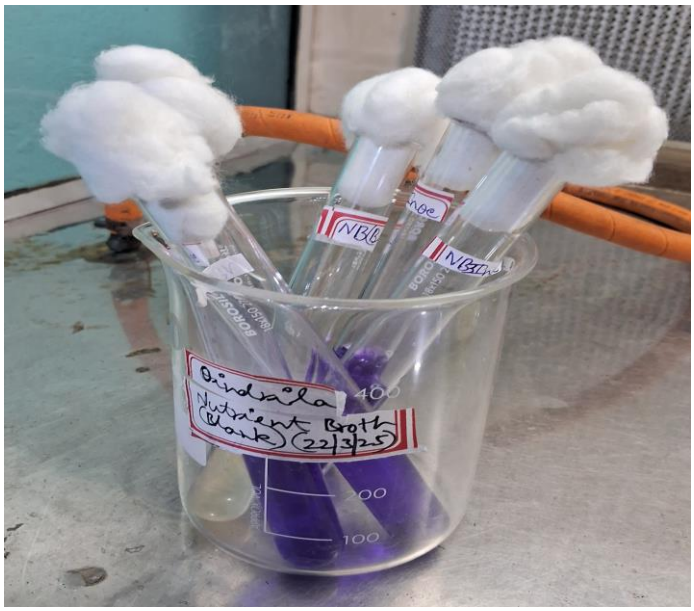
Pictures of Inoculated and Blank Nutrient Broth Test Tubes.

Result and Observation of Experiment- Biofilm Estimation:

After performing Biofilm Estimation, we would keep all 4 Test tubes in test tube rack marked as NB1(Inoculated), NB2(Inoculated), NB3(Inoculated), NB4(Inoculated) Test tubes and we could see that few Bacterial cells and less amount of Crystal Violet remains inside these 4 Nutrient Broth Test Tubes. We could also see that most of the Biofilm had occurred in test tubes marked as Nutrient Broth3 and Nutrient Broth4. Whereas, less amount of Biofilm had occurred in test tubes marked as Nutrient Broth1 and Nutrient Broth2. Then we would discard these 4 Nutrient Broth test tubes and that Conical Flask having Nutrient Broth and inoculum which we poured from those 4 test tubes in this Conical Flask. Then we would take inoculum from EMB Agar Streaked Plate1 and inoculate in that new Conical Flask and keep the Conical Flask inside Incubator at 37°C for 78 hours. Then we would wrap that EMB Agar Streaked Plate1 with Parafilm and keep that plate inside Fridge at 4°C.

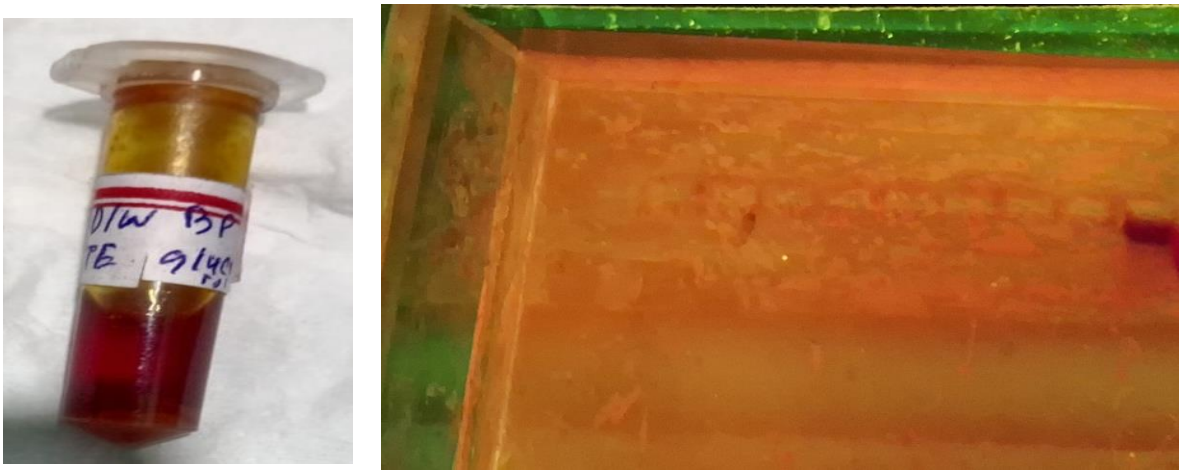
All the pictures of Biofilm Estimation.




Nutrient Broth
(Blank) (22/3/25)

Result and Observation of Experiment- Plasmid DNA Isolation:-

By putting the tray inside UV Transilluminator, we would observe that the sample was not glowing. From this we could understand that my sample was not containing Plasmid DNA and that's why, my sample was not glowing. So, I got the result of Plasmid DNA as Negative Test in my Sample. Pictures of Loading Dye and after Sample Loading the Electricity is applied.



Picture of Plasmid DNA Isolation

Results and Discussions:

From my Project Dissertation, I have got a perfect result on the Project Thesis Experiment:- "Isolation and Growth Study of Coliform Isolated From Prayagraj With Antimicrobial Peptide". We performed many practicals or experiments on this Project Thesis Topic. The Practicals or Experiments which we performed and got perfect results are:- Bacterial Pure Culture Isolation, Performing Bacterial Gram Staining, EMB Agar Plating, Nutrient Agar Medium Plating, Experiment of Bacterial Inhibitory Zone, IMViC Test, Bacterial Inoculation in Nutrient Broth Test Tubes, Biofilm Estimation, Bacterial Plasmid DNA Isolation, etc. We can understand that, by taking pure culture from Prayagraj, and streaking in Nutrient Agar Plate and then keeping those plates inside Incubator for atleast 24 hours. After 24 hours, we can see the presence of Bacterial Colonies in Nutrient Agar Streaked Plates. Then we would

perform Gram Staining with these bacteria , and saw the structure of Bacterial Colonies at 40X in Light Microscope. Rest in all other practicals, we could see the presence of Coliform Bacteria. But, unfortunately we did not get any Plasmid DNA from my 1 sample.

Conclusion:

A conclusion for a study on "Isolation and Growth Study of Coliform to Perform IMViC Test, Biofilm Estimation and Plasmid DNA Isolation" would integrate the findings from each of these distinct but interconnected analyses to provide a holistic understanding of the isolated coliform strains.

In conclusion, this study provides a foundational characterization of coliform isolates through a combination of phenotypic and genotypic analyses. The observed prevalence of biofilm formation and plasmid carriage underscores the importance of continued surveillance and comprehensive strategies to mitigate the potential threats posed by these ubiquitous and adaptable microorganisms.

Appendix:

The Practicals or Experiments which we performed and got perfect results are:- Bacterial Pure Culture Isolation, Performing Bacterial Gram Staining, EMB Agar Plating, Nutrient Agar Medium Plating, Experiment of Bacterial Inhibitory Zone, IMViC Test, Bacterial Inoculation in Nutrient Broth Test Tubes, Biofilm Estimation, Bacterial Plasmid DNA Isolation, etc.

Future Directions:

Future research should focus on specific next steps, e.g., sequencing the identified plasmids to determine the specific genes they carry, especially those related to antibiotic resistance and virulence, investigating the correlation between specific IMViC patterns and biofilm-forming capacity, exploring the impact of environmental factors on biofilm formation and plasmid transfer, conducting detailed growth kinetics under different stress conditions. Such investigations would provide deeper insights into the epidemiological significance and potential health risks posed by these coliform populations.

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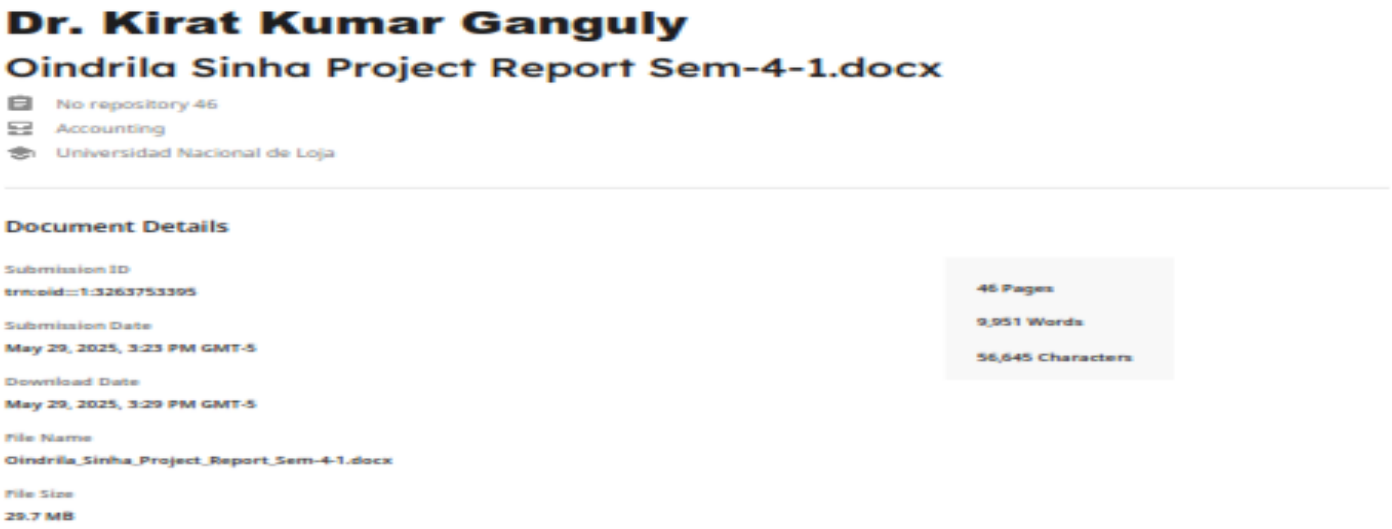

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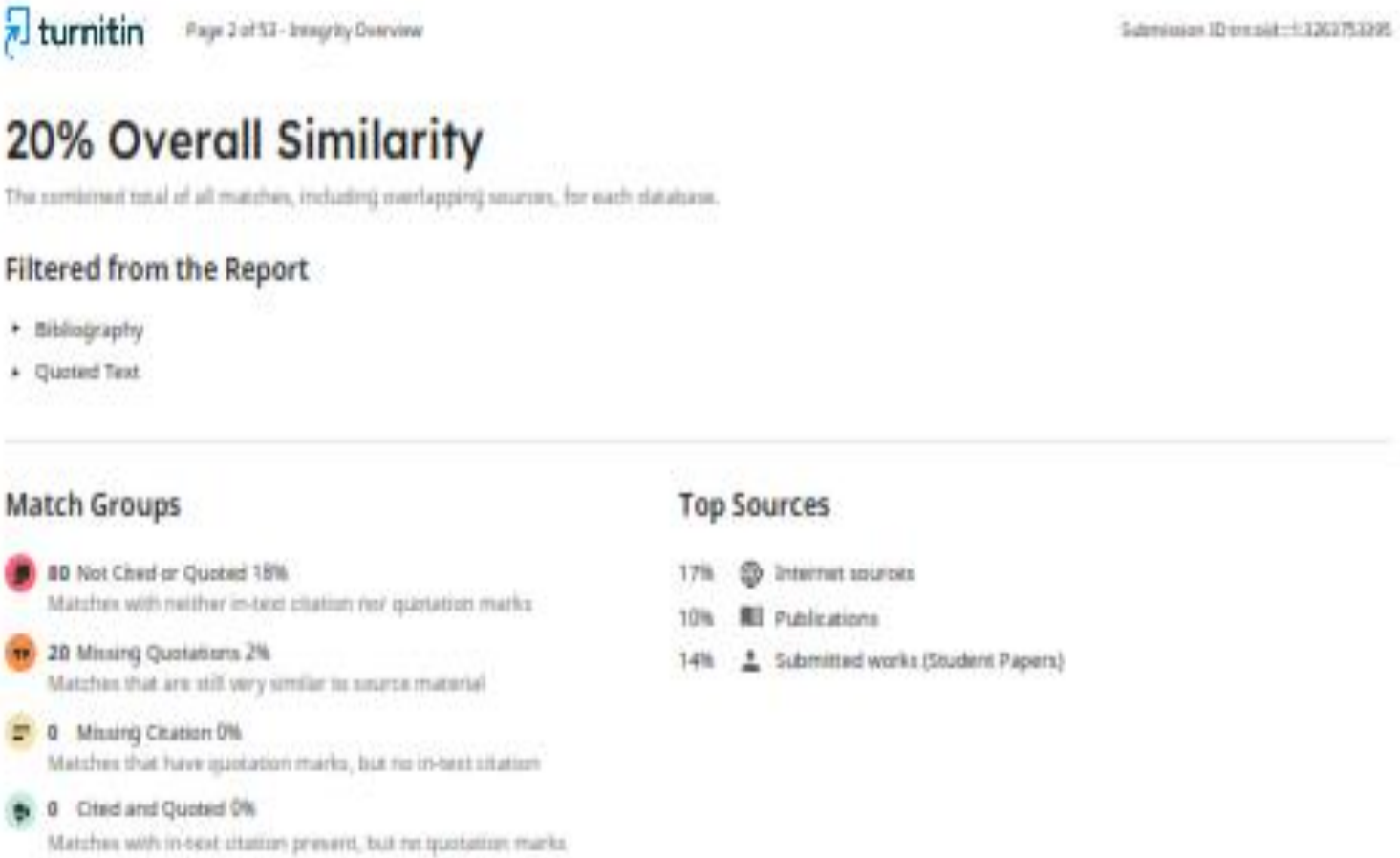

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